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TITLE: Riboswitch-Mediated Aptamer Binding for Imaging and Therapy (RABIT): A Novel Technique to Selectively Target an Intracellular Ligand Specific for Ovarian Cancer

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14. ABSTRACT We have proposed a novel technique to image and treat ovarian cancer with very high specificity, low background for imaging and low toxicity for therapy. We will make a riboswitch consisting of two aptamers and a sensor region that can hybridize with the specific intracellular ovarian cancer cell marker, VICKZ. The target for the first aptamer, EpCAM, is a surface antigen that is up-regulated in cancer cells. During cellular recycling, EpCAM will carry the attached riboswitch into the cell. Inside the cell, the riboswitch will interact with VICKZ mRNA. This interaction will change the conformation of the riboswitch to expose a second aptamer in the correct conformation to bind an administered radioactive agent which rapidly enters and exits the ovarian cancer cells, eg. antipyrine. Depending on the radioactive isotope attached to the agent, the ovarian cancer cells will be either imaged (¹²³ I) or killed (¹³¹ I). We have successfully made both DNA and RNA aptamers to antipyrine, the 2 nd proposed aptamer, and have been working on isolating aptamers to iodoantipyrine. We have also accomplished the important task of introducing a large portion of the eventual riboswitch into the cell interior where it appears to binds selectively to the target cancer cell marker, VICKZ mRNA.					
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Revised Final Progress Report

We have revised the Final Progress Report to conform to the comments of the reviewer. Revisions are written in red. Below, in blue are the reviewer's specific comments and our response to them.

No progress was discussed for Tasks 2 and 5, even though they were noted in earlier reports. Because of research delays as a result of the T3 and T4 aptamers not being useful, we changed the tasks in the SOW in our previous Progress Reports as the reviewer has noted, in order to more efficiently progress to the overall goal of the proposed research. We also incorrectly labeled task 5 in our Final Progress Report. We have now corrected the tasks in the final progress Report with an explanation in red for each task.. Task 2 has been included with Task 1 since it describes different methods of isolating and testing binding of the RNA and DNA aptamers selected by each of the collaborating laboratories.

The report provided only example data that did not support all research accomplishments or provide supporting data to facilitate an independent interpretation of results. ex. the report outlines results from screening DNA sequences, but doesn't describe the final sequences.

We have listed within the body of the report the ten most abundant DNA aptamer sequences, derived from the most common motif. These sequences were synthesized and their binding tested as explained in the report. We have added this material in red to the Final Progress Report.

No work initiated for Task 3, needs to be explained.
See revised Final Progress Report section in red

Report describes screening aptamer libraries for recognition of antipyrine, MUC1 and aminoantipyrine, but never T3 or T4 as originally intended and described in the SOW.

Please see page 4 of our Final Progress Report in the first paragraph of the BODY, wherein we explain that we were unable to use the published T3 and T4 aptamer. The aptamer does not bind T3 and T4 in our hands. In addition, since no additional publications have appeared describing this aptamer, it is likely that other laboratories have been unsuccessful in using this aptamer. Because of our inability to show binding with this aptamer after several months we turned to the use of antipyrine, for which we successfully showed aptamer binding as described in our Final Progress Report. We reproduce that paragraph below:

Tasks 1-3 – Optimization and calibration of an aptamer that binds a radioactive target

Our original research strategy describes the use of a thyroid hormone aptamer as the aptamer exposed by action of the riboswitch. This aptamer was initially selected because thyroid hormone is readily taken up by most mammalian cells, including BAT cells. It can also be readily labeled with different isotopes of iodine for imaging and quantitating UCP1. In addition, an aptamer to thyroid hormone, with a description of its isolation, had already been published prior to the time of our proposal. We worked for several months using the RNA oligonucleotide described as an aptamer to thyroid hormone that contained modified nucleotides to provide RNase resistance, but we were unable to show specific binding to either T3 or T4

thyroid hormone. However, we did show cell uptake of a fluorescently labeled construct containing an aptamer to EpCAM another cell surface marker joined to the reported thyroid aptamer.

Since the reported thyroid hormone aptamer was not functioning in our hands, we decided to build our riboswitch with an aptamer to an alternate target. We turned to antipyrine as a good candidate for the second aptamer in the riboswitch, which would be exposed upon riboswitch binding to VICKZ mRNA. Antipyrine can be readily labeled with isotopes of iodine, rapidly diffuses in and out of cells, and has a short biologic half-life. Since there are no published reports of an antipyrine aptamer we have been forced to isolate our own antipyrine aptamer. Because of these features of antipyrine, the development of an antipyrine aptamer appears to have a wide potential in the identification of any intracellular cell marker, including cancer cell markers, using the novel riboswitch methods we have proposed.

INTRODUCTION

We have proposed a novel technique to image and treat ovarian cancer with very high specificity, low background for imaging and low toxicity for therapy. We proposed to make a riboswitch consisting of two aptamers and a sensor region that can hybridize with the specific intracellular ovarian cancer cell marker, VICKZ. The target for the first aptamer, EpCAM, is a surface antigen that is up-regulated in cancer cells. During cellular recycling, EpCAM should carry the attached riboswitch into the cell. Inside the cell, the riboswitch will interact with VICKZ mRNA. This interaction will change the conformation of the riboswitch to expose a second aptamer in the correct conformation to bind an administered radioactive agent which rapidly enters and exits the ovarian cancer cells, eg. antipyrine. Depending on the radioactive isotope attached to the agent the ovarian cancer cells will be either imaged (^{123}I) or killed (^{131}I). We have successfully made both DNA and RNA aptamers to antipyrine the 2nd proposed aptamer, and have been working on isolating aptamers to iodoantipyrine. We have also accomplished the important task of introducing a large portion of the eventual riboswitch into the cell interior where it appears to bind selectively to the target cancer cell marker, VICKZ mRNA.

BODY

Task 1 and 2– Optimization and calibration of an aptamer that binds a radioactive target

In our original SOW task 1 was stated as follows: **Optimizing the T3 aptamer.** However, as explained below and in previous Progress Reports, we have had to select a different potential radioactive target and an aptamer to that target. Following the selection of the RNA and DNA aptamers we next tested their binding to antipyrine.

Our original research strategy describes the use of a thyroid hormone aptamer as the aptamer exposed by action of the riboswitch. This aptamer was initially selected because thyroid hormone is readily taken up by most mammalian cells, including BAT cells. It can also be readily labeled with different isotopes of iodine for imaging and quantitating UCP1. In addition, an aptamer to thyroid hormone, with a description of its isolation, had already been published prior to the time of our proposal. We worked for several months using the RNA oligonucleotide described as an aptamer to thyroid hormone that contained modified nucleotides to provide RNase resistance, but we were unable to show specific binding to either T3 or T4 thyroid hormone. However, we did show cell uptake of a fluorescently labeled construct containing an aptamer to EpCAM another cell surface marker joined to the reported thyroid aptamer.

Since the reported thyroid hormone aptamer was not functioning in our hands, we decided to build our riboswitch with an aptamer to an alternate target. We turned to antipyrine as a good candidate for the second aptamer in the riboswitch, which would be exposed upon riboswitch binding to VICKZ mRNA. Antipyrine can be readily labeled with isotopes of iodine, rapidly diffuses in and out of cells, and has a short biologic half-life. Since there are no published reports of an antipyrine aptamer we have been forced to isolate our own antipyrine aptamer. Because of these features of antipyrine, the development of an antipyrine aptamer appears to have a wide potential in the identification of any intracellular cell marker, including cancer cell markers, using the novel riboswitch methods we have proposed.

Isolating an RNA aptamer that recognizes antipyrine

We have used a novel approach to isolating aptamers by utilizing Surface Plasmon Resonance (Biacore) in a preparative, rather than analytic, manner, in collaboration with Dr. Alex Varvak at Bar Ilan University. Amino antipyrine was attached to carboxylated Biacore chips using standard chemistries. The template library for synthesizing the aptamers was constructed as follows: fixed sequences were chosen for the 5' and 3' ends that would allow for easy sequencing on a MiSeq sequencer. The 33 nucleotides separating the 5' and 3' ends were completely random, yielding a library with a predicted complexity of over 10^{14} . RNA was synthesized from the template library in the presence of 2'-fluoro-UTP and 2'-fluoro-CTP, in order to stabilize the transcripts against RNase degradation. A mutant T7 RNA polymerase, which allows efficient incorporation of the modified nucleotides (a gift from Dr. James McNamara at the University of Iowa), was used to synthesize the RNA transcripts. Transcripts were used as analytes to bind to the antipyrine-linked Biacore chips. After thorough washing of the chip with saline, transcripts were eluted off and collected using a high concentration of antipyrine. cDNA was generated from the eluted RNAs, amplified in a limited fashion, and then used again as templates for RNA synthesis. A total of three rounds of selection were performed, after which the eluted RNA was sequenced on a MiSeq sequencer. Dr. Yael Altuvia and Professor Hanna Margalit identified sequences that were highly enriched (almost 1000-fold) by first identifying a set of -mers (from 12 nucleotides long or longer) that were enriched, and then using DNA clustering and motif searches to identify sequences that were consistently enriched in the eluted RNAs. From this analysis, 6 candidates were chosen for validation.

Initially, candidates were validated using the same Biacore chips on which they were isolated. As seen in figure 2A, 5 out of 6 of the candidates show clear binding to the antipyrine-linked chip; binding is observed as an increase in the curve over time (after the initial jump when the sample enters the chamber). Even more encouraging is the fact that this binding can be abrogated by incubating the aptamers first in a solution with soluble antipyrine (figure 2B). In this case, the absence of binding is observed from the fact that the curves are all flat, with no increase in the curve over time, as was seen in figure 2A.

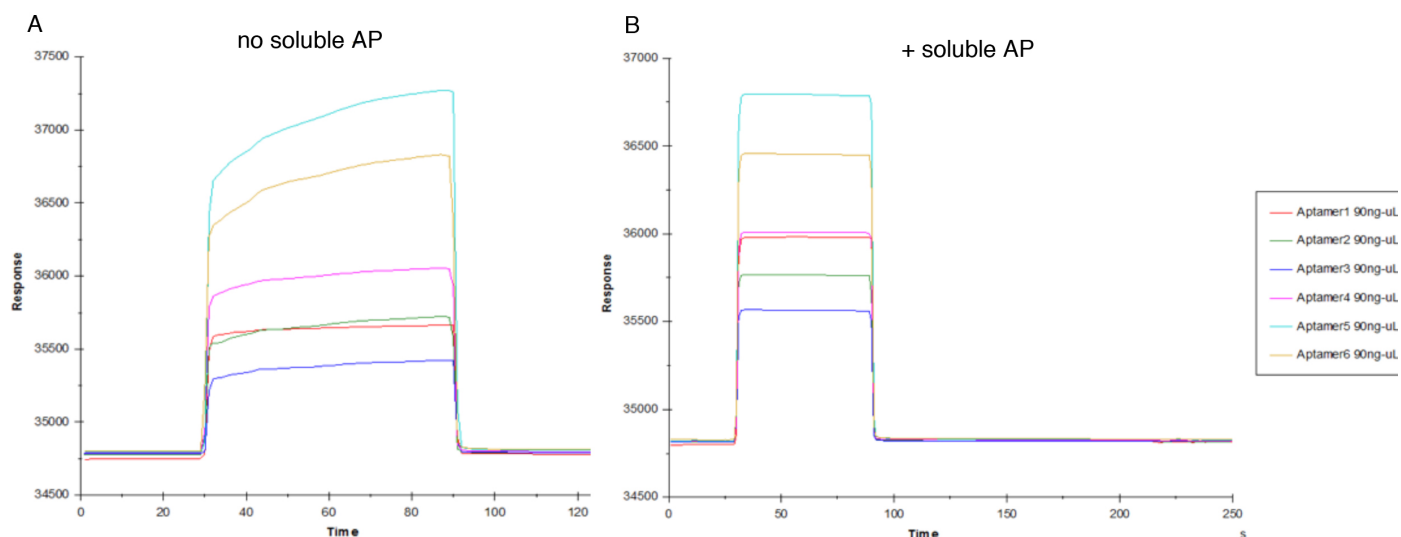


Figure 2. Sensorgram from Biacore analysis of antipyrine aptamer candidates

4 out of the 6 antipyrine aptamer candidates were chosen for further validation using fluorescence activated cell sorter (FACS) analysis. Candidates were synthesized as above and then incubated with beads to which amino antipyrine had been linked (also using carboxylated beads, but attached with a different carbon chain), or to control beads without linked antipyrine. Bound aptamer was detected by incubation with a fluorescent DNA probe that hybridizes to the 3' fixed sequence of the aptamer, and analyzed by FACS.

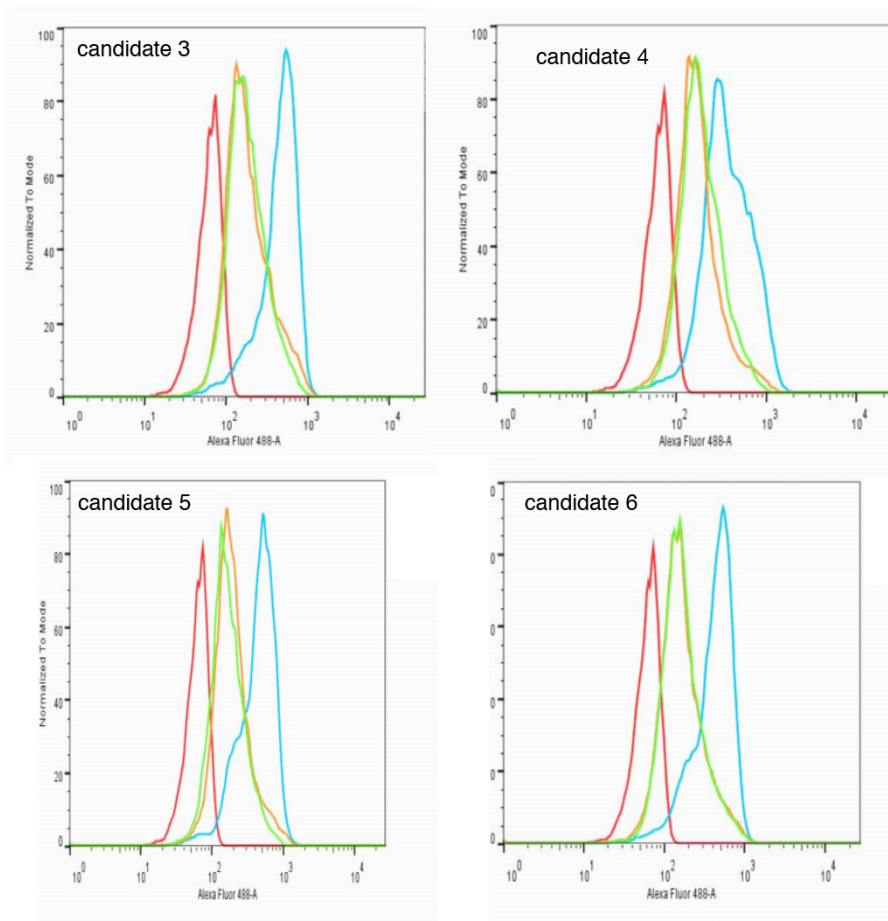


Figure 3.
Validation of the
antipyrene aptamers
using FACS.

Candidate aptamers 3-6 were synthesized in vitro in the presence of 2'-fluoropyrimidines and incubated with beads that were either control beads (red curves) or beads linked to antipyrene (blue, green and orange curves). A fluorescent DNA probe complementary to the 3' end of the aptamers was hybridized to the aptamers and then passed through the FACS. In the case of the green and orange curves, either 1000-fold (orange) or 2000-fold excess (green)

soluble antipyrene was added to the aptamers before incubation with the beads. In all cases, addition of the aptamer caused a marked increase in fluorescence of the beads, and the soluble antipyrene competed for the binding.

As can be seen in the FACS curves, all four of the aptamers bound the antipyrene-linked, but not the control, beads (observed as a rightward shift in the blue curve relative to the red curve), and this binding could be competed by soluble antipyrene. (Increasing antipyrene concentrations were not effective at completely competing for binding, perhaps because of solubility and aggregation problems.) In two of the aptamers, there are 13 nucleotide-long sequences that are virtually identical. Currently, we are looking at mutants of these sequences to see if we can better identify which sequences are important for the binding.

Isolating an RNA aptamer using Cell-SELEX that recognizes cleaved MUC1 on the surface of cells

We have used a Cell-SELEX approach, coupled with deep sequencing and bioinformatics, to identify aptamers recognizing MUC1. We obtained NIH 3T3 cells that were transfected with a MUC1 plasmid encoding the peptide that undergoes self-cleavage and reassociation from Prof. Danny Wreschner at Tel Aviv University. 2'-fluoropyrimidine-modified aptamers were synthesized from the same random library described above. Aptamers were first incubated with the (non-transfected) parental cell line in order to subtract aptamers that recognize other cell surface markers. The supernatant not associating with the cells was then placed on cells

transfected with MUC1 and allowed to endocytose for 20'. Cells were rinsed well, lysed, incubated with RNase A to digest cellular RNA, and then the resistant RNA was reversed transcribed and limited amplification performed to generate template for the next round. After three rounds of subtraction followed by selection, the population of selected aptamers was tested for its ability to bind MUC1-expressing cells. The aptamers were incubated with either parental or MUC1-expressing cells that were inhibited by sodium azide from undergoing endocytosis, and then binding was assayed by FACS as described in (1). As seen in figure 4, a clearly visible fraction of the cells were bound by aptamers, detected as a shoulder shifted to higher fluorescence. These results encouraged us to analyze the aptamer population as was done with the antipyrene aptamers (section 1). Much to our surprise, 60% of the almost 1.5 million reads obtained from the third round selection were the same sequence. This could certainly explain the shoulder seen in figure 4. We are now in the process of verifying candidates from this selection.

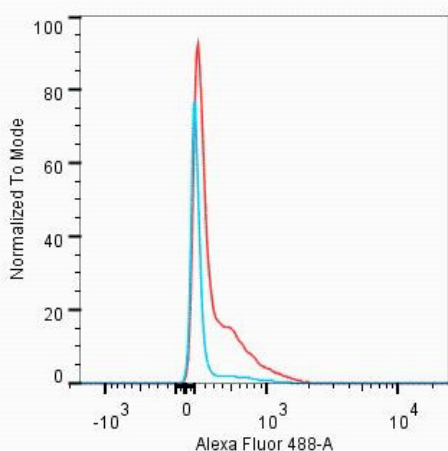


Fig. 4. Analysis of the population of aptamers selected for MUC1 binding after 3 rounds of selection.

The entire population of aptamers after three rounds of selection were incubated with either the parental, NIH 3T3 cells (blue curve) or with MUC1-transfected NIH 3T3 cells (red curve) and analyzed as described in figure 3. The cells were prevented from endocytosing the bound RNAs by treatment with sodium azide. A population of cells bound by aptamers is observed as a shoulder of higher fluorescence on the red curve.

Meanwhile, to avoid the use of modified nucleotides, the Kolodny lab began a search for DNA rather than RNA aptamers to antipyrene. A modified SELEX approach was used, followed by next generation sequencing. Using limited SELEX rounds on magnetic beads, followed by multiple parallel next generation sequencing we have successfully selected ssDNA aptamers showing highly selective binding of the aptamers to antipyrene.

Antipyrene is a small molecule (MW 188) which provides particular challenges for aptamer selection. We need an agent that rapidly enters and leaves normal cells and is only retained in cells with an exposed aptamer to the agent. Any other agent we would choose that rapidly enters and leaves cells would also have to be small. Since antipyrene in the doses we will be using is known to be nontoxic and readily labeled with radioactive iodine we have chosen to proceed with selection of an aptamer to this small molecule in spite of the known difficulty of selecting aptamers for small molecules.

The major difficulty with selecting aptamers for small molecules arises from the fact that aptamers are usually selected by binding one portion of the target, such as a protein, to a solid support such as magnetic beads. Another portion of the molecule far removed from the solid support binding site is then free to bind to aptamers during the SELEX selection process. With a small molecule such as antipyrene the binding of the molecule to the solid support often changes the conformation of the target or makes the molecule inaccessible to the potential aptamers. After considerable work with changes in binding sites, magnetic beads and linkers we were able to select aptamers to antipyrene by using aminoantipyrene conjugated with a carboxyl terminated linker on magnetic beads.

Aminoantipyrene was linked to carboxyl groups on side arms of superparamagnetic beads. After incubation of the antipyrene linked beads with a library of approximately 10^{14} 40

base random DNA sequences, bracketed with distinct, fixed 5' and 3' primers, the beads were extensively washed. The attached oligonucleotides were then eluted, amplified by PCR for only 15 rounds to reduce selection of only those oligonucleotides showing preferential PCR amplification, and used in a subsequent round of aptamer selection. After 5 rounds of SELEX selection, the resulting sequences had adapters and index sequences added for sequencing using an Illumina next generation massively parallel sequencer at the Hebrew University in Jerusalem, Israel, the home institution of the Yisraeli lab.

Data from the sequencer was analyzed using the Genome Tools tallymer software for counting, indexing and searching k-mers, obtained on the internet from Stefan Kurtz at the Center for Bioinformatics at the University of Hamburg in Hamburg, Germany. From the Illumina sequencer we analyzed separately sequences of lengths 30, 25, 20, 17, 16, 15, 14, 13, 12 and 11 nucleotides. For each k-mer we searched for sequences appearing greater than 100 times. K-mers containing adaptor or primer sequence, or containing long runs of a single nucleotide were eliminated. We obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) oligonucleotides corresponding to the ten most frequently appearing sequences for each k-mer (all of which were 15 nucleotides or smaller in length). **These aptamers had the following sequences:**

13mer1	GGTGGTGTGTGGG
13mer2	GTGGGAGGCGGGG
13mer3	GGTGGTGTGTGGG
13mer4	GTGTGTGGGGCAG
13mer5	CGGGTGGTGGAGT
12mer1	GGGGTTAGTGTG
12mer2	TTGGTGTGGTG
12mer3	TGTGTGGGGCAG
12mer4	CGGGTGGTGGAG
12mer5	GTGGGGTTGGAG
12mer6	TTGGTGGTGTGT
12mer7	TGTGTGGGGCTG

The oligonucleotides were labeled with ^{32}P at the 5' end and then tested in a binding assay with aminoantipyrine joined to magnetic beads. Figure 2 shows the results of a binding assay with 12mer2, which showed a particularly low binding constant as analyzed by GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California USA, www.graphpad.com.

The dissociation constant using one site saturation averaged over 3 experiments was calculated as 3.9 ± 0.8 nM.

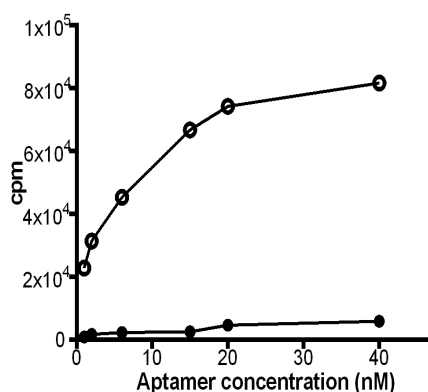


Fig. 2 Binding kinetics of antipyrine and aptamer Radiolabeled 12mer2 aptamer at increasing concentrations was applied to antipyrine linked to magnetic beads. The beads were washed and counted. The calculated dissociation constant was 3.9 ± 0.8 nM. Open circles are cpm averaged over 3 experiments for aptamer binding to antipyrine linked to magnetic beads. Closed circles are cpm averaged over three experiments for binding to magnetic beads alone

To confirm that the sequences we had chosen were suitable aptamers, we next performed competition assays with antipyrine, aminoantipyrine and iodoantipyrine. In particular we wanted to be sure that we had selected at least one or more aptamers that would bind iodoantipyrine, since we intended to eventually use antipyrine labeled with radioactive iodine isotopes as a target for our aptamer. The targets were combined separately to each aptamer candidate and then incubated with the bead bound antipyrine. Assays with antipyrine and aminoantipyrine showed good competitive binding. However the iodoantipyrine competition assays with each of 20 candidate aptamers showed limited competition with the antipyrine bound to the beads. Because control experiments using the carboxylated beads without attached antipyrine showed very low binding of radiolabeled aptamers to iodoantipyrine, it would appear that the iodine moiety on the iodoantipyrine is interfering with the binding of the candidate aptamers.

The difficulties and challenges of isolating aptamers to small molecules is well known (McKeague and DeRosa 2012, Kim and Gu 2014). Binding of a very small target to a solid support in order to isolate aptamers can interfere with binding of aptamers to the target or change the conformation of the small molecule such that aptamers bound to the target attached to the solid support are not true aptamers for the target in solution. Moreover, in other cases with targets bound to a solid support, a SELEX selected aptamer may be an aptamer to the link between the target and the solid support, rather than the target itself.

Ideally, the binding of target and aptamer should be performed in solution to maintain the solution structure of both aptamer and target. We propose two approaches to select aptamers for the small molecule iodoantipyrine. In both cases, both aptamer and target first bind in solution, rather than have the target bind to the library attached to a solid support. In our first approach to selection of DNA aptamers to IAP we have mixed library and the target IAP interact in solution during each round of aptamer/target binding. We used a ssDNA library of approximately 10^{14} molecules consisting of two random regions of the DNA, separated by a fixed capture sequence, and fixed sequences at the 5' and 3' ends of the DNA serving as primer sites for PCR amplification. Biotinylated DNA containing the complementary docking sequence was attached to streptavidin coated magnetic beads. After incubation of the library and target IAP in solution the beads containing the linked complement to the docking sequence was added to the solution. DNA that had not interacted with the target and maintained its conformation had presumably bound to the complementary docking sequence attached to the magnetic beads. DNA binding to the target had a changed conformation so that the docking sequence was no longer able to bind to the complementary sequence linked to the beads. After magnetic collection of the beads, aptamers binding the target and remaining in the supernatant were removed. The aptamers were amplified with PCR using a 3' biotinylated primer, and then attached to streptavidin beads. The strands were separated at high pH and the ssDNA sense sequence amplified by sequential rounds of target and aptamer incubation in solution followed by magnetic collection and amplification of the DNA not bound to the target.

After 5 rounds of selection and amplification, the resulting aptamers are currently being sequenced with high throughput Next Generation sequencing at the Harvard Biopolymers facility to identify high frequency binding motifs. We will be assisted in the analysis of the sequencing data by the Harvard School of Public Health Bioinformatics Core Laboratory. Motif sequences from the control SELEX will be subtracted from the IAP selected sequences during the analysis. We will confirm that we have selected aptamers with high binding to IAP by incubating the presumptive biotinylated aptamers attached to streptavidin beads with ^{14}C iodoantipyrine (Perkin Elmer) and confirming high binding of the ^{14}C IAP to the presumptive aptamers. After confirming high binding with radiolabeled IAP we will confirm competitive binding with nonradioactive IAP.

Graphene oxide (GO) will adsorb ssDNA but not dsDNA (Park et al 2012). In a further attempt to isolate aptamers to the very small IAP molecule, We have bound a library of random

ssDNA oligonucleotides to GO. The GO with bound DNA was then exposed to the target IAP. Aptamers to the target fold around the target, become partially dual stranded and lose their attachment to the GO. We collected the GO by centrifugation and amplified the target bound aptamers in solution with PCR using a biotin labeled primer for the reverse strand. The dsDNA PCR products were then attached to streptavidin beads. The DNA strands were separated with high pH. After removal and neutralization of the alkaline solution, the forward ssDNA was again adsorbed on GO and again exposed to fresh target molecules.

After 5 rounds of selection and amplification, the resulting aptamers are now being sequenced with high throughput Next Generation sequencing.

Task 3– Testing the ability of a surface aptamer to direct delivery of attached molecules into cells

Task 3 of our SOW describes: **Optimizing retention within cells of I^{125} -T3 by the EpCAM/T3 DA.** Since we had fallen behind in our schedule because of our fruitless testing of the published aptamer to T3 and T4, we decided that while selecting an aptamer to antipyrine we could efficiently test uptake of other components of our proposed riboswitch. We therefore began testing the ability of a surface aptamer to direct delivery of attached molecular components of the riboswitch. Task 3 was thus converted to a related task, for which, as we describe below, yielded excellent results applicable to our overall goal.

As proof of concept that an aptamer can mediate cell uptake into cultured cells, ES2 ovarian cancer cells were exposed to a DNA EpCAM aptamer joined to Cy3-labeled DNA. Figure 3, using confocal microscopy, demonstrates increased cytoplasmic signal in cells exposed to the construct (compared to background fluorescence in control cells), indicating that the EpCAM aptamer and associated sequences is specifically endocytosed by these cells. The presence of the fluorescence label only in the cytoplasm, and not in the nucleus, suggests that this construct has not been degraded.

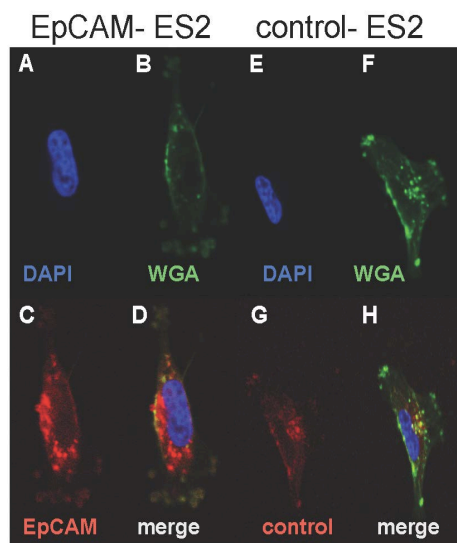


Fig. 3. Uptake of EpCAM/antiVICKZ aptamer. Fluorescently labeled antiVICKZ DNA joined to EpCAM aptamer was incubated with ES2 cells. Uptake was assayed by confocal microscopy. A-D, ES2 cells incubated with 1.8uM EpCAM-antiVICKZ labeled with texas red fluorophore. E-H, ES2 cells with no construct added. DAPI (4',6-diamidino-2-phenylindole) stained nuclei are seen in blue, WGA (wheat germ agglutinin) stained cell membranes are seen in green. Most of the ES2 cells incubated with the antiVICKZ DNA/EpCAM aptamer show increased red cytoplasmic staining compared to control cells (that show only background fluorescence), indicating specific uptake of the EpCAM-containing construct.

Fluorescence activated cell sorting (FACS) analysis, (fig. 4) also demonstrated that the EpCAM aptamer and associated sequences is efficiently endocytosed by essentially all of the cells.

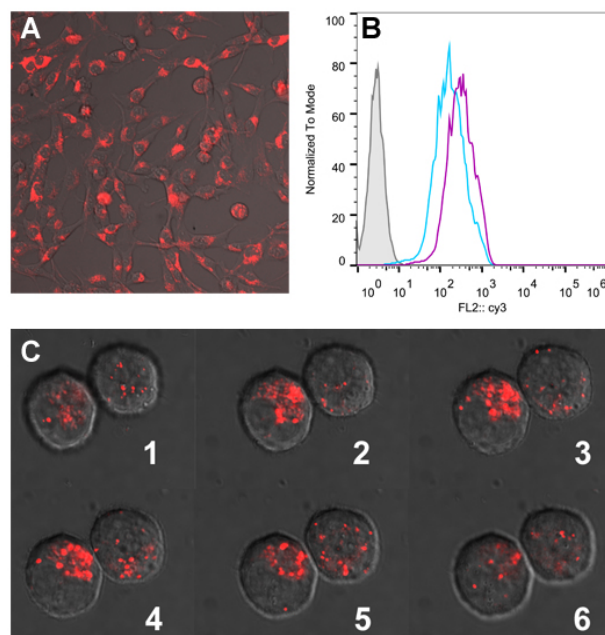


Fig. 4 Uptake of EpCAM aptamer. Fluorescently labeled DNA oligonucleotide linked to EpCAM aptamer was incubated with ES2 cells. A. ES2 cells were incubated with 200nM EpCAM-oligonucleotide labeled with Cy3. Uptake was observed by confocal microscopy (40X). B. FACS analysis was used to quantify uptake of the Cy3-EpCAM-oligonucleotide. ES2 cells were incubated overnight with no (gray), 50 nM (blue), or 200 nM (purple) Cy3-EpCAM-oligonucleotide, trypsinized, and analyzed for forward scatter and fluorescence intensity. Essentially all of the cells showed uptake of the oligonucleotide at both concentrations. C. Z-stack analysis (1-6, sections spaced 2 microns apart) of ES2 cells incubated with 200 nM Cy3-EpCAM-oligonucleotide clearly shows the presence of the label within the cell and not on the cell membrane (300X).

Task 5 – Testing the ability of an EpCAM aptamer/antiVICKZ oligo to be internalized and recognize VICKZ mRNA

Our original SOW describes task 5 as: **Creating an EpCam-VICKZ-T3 CO**. However, because of delays caused by our having to select aptamers to antipyrine and confirm their binding to antipyrine we were unable to progress to actually constructing our proposed riboswitch in the time allotted in this contract. We therefore proceeded, in tandem with our work on selecting antipyrine aptamers, to confirm that we could internalize an anti VICKZ oligomer that would be a part of the eventual riboswitch. We also wished to show that once inside the cell it would suppress cell motility for which VICKZ is necessary. In previous Progress Reports we have changed the description of task 5 to better represent our progress.

The VICKZ family of proteins are associated with cell migration and movement, are abundant in many embryonic and cancer cells and help mediate ES2 migration in vitro. Having shown that the EpCAM aptamer can direct uptake into ES2 cells, we next wanted to assess the ability of the antiVICKZ sequence in this oligonucleotide to recognize and bind endogenous VICKZ RNA. We reasoned that if the DNA antiVICKZ sequence would hybridize to endogenous VICKZ RNA, the hybridized RNA would be degraded by endogenous RNase H, leading to a VICKZ knockdown and inhibition of migration. ES2 ovarian cancer cells in culture were exposed to the EpCAM/antiVICKZ3 construct. The cells were then transferred in serum free media into Boyden chambers (tissue culture inserts with polyethylene terephthalate membrane of pore size 0.8µm) in wells of serum-containing media. The established chemoattractive gradient caused the cells to migrate through the membrane toward the serum-containing media. On inspection and cell counting of up to 10 microscopic fields for each assay, the treated cells showed significant inhibition of migration compared to control untreated cells. On repeated assays the number of treated cells that migrated through the membranes was 30-50% lower than the number of untreated cells that migrated. In figure 5, control cells showed 667 cells migrating through the membrane in 10 high power microscopic fields, while the construct treated cells showed only 336 cells in ten microscopic fields. As a control for nonspecific effects of DNA either on the exterior or interior of the ES2 cells, we incubated the ES2 cells with a construct

consisting of the EpCAM aptamer joined to a nonspecific DNA sequence. There was no statistically significant difference between the control cell migration and the migration of cells incubated with this construct. These results indicate that the EpCAM aptamer –anti VICKZ oligonucleotide inhibits cell migration across a membrane specifically due to the anti VICKZ sequences. We suggest that this argues for the ability of the antiVICKZ sequences to recognize and bind endogenous VICKZ RNA. Western blot analysis of extracts from the treated ES2 cells shows a 50% reduction in VICKZ3 protein expression compared to extracts from untreated control cells, using a pan-VICKZ antibody (data not shown). We are currently testing the specificity of this knockdown using antibodies that specifically recognize VICKZ1, 2, or 3, and chimeras of EpCAM aptamers with anti-VICKZ1, 2, and 3 antisense sequences.

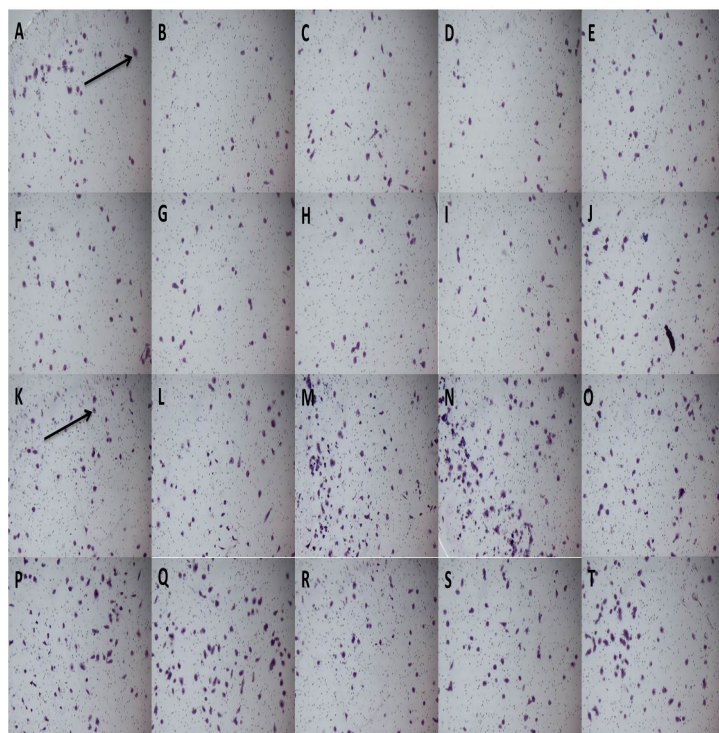


Figure 5. Migration of ES2 ovarian cancer cells. ES2 ovarian cancer cells in culture were allowed to adhere overnight to tissue culture plates and were then exposed to 1.6 uM EpCAM aptamer/antiVICKZ (panes A-J) or no construct (panes K-T) for 2 hours. The cells were trypsinized, neutralized with serum containing media, centrifuged, resuspended in serum-free media and then placed onto Boyden chambers in wells of serum-containing media for 5 hours. Non-invading cells were removed and cells were fixed and stained with Toluidine Blue. Each condition was examined under 100X magnification and cells from ten random fields were counted. Arrow in A points to cell. Arrow in K points to membrane pore. The microscopist performing the cell counting was blinded to the specific treatments. Ten ROI's were selected for each sample that encompassed the entire field.

Besides confirming that at least a portion of the eventual riboswitch could be taken up by the ES2 cells in culture, these

results suggested to us the possibility of using the EpCAM/antiVICKZ DNA construct to limit cell (eg. cancer cell) mobility and metastasis of treated cells. The VICKZ family of proteins includes 3 major families of proteins. Our experiments thus far have focused on only VICKZ 3. We are now exploring the use of EpCAM aptamer joined to antiVICKZ sequences for VICKZ 1 and VICKZ 2 and a combination of all three constructs together in ES2 migration assays. If these experiments demonstrate decreased cell migration greater than 90% we will test inhibition of invasion through matrigel in the Boyden chamber and perform further control experiments before embarking on animal experiments.

KEY RESEARCH ACCOMPLISHMENTS

- * **Identification of highly specific cell surface markers for brown fat cells**
 - Selection of candidate antipyrine modified RNA aptamers
 - Selection of candidate antipyrine DNA aptamers
 - Selection of candidate MUC1 modified RNA aptamers
 - Determination of binding constants of DNA aptamers
 - Demonstration of the intracellular uptake of constructs of EpCAM and portions of the riboswitch

- Demonstration of the EpCAM aptamer/antiVICKZ oligo portion of the riboswitch to recognize and hybridize with VICKZ mRNA
- Dramatic reduction in cell migration with EpCAM aptamer/antiVICKZ treatment with cells in culture.

REPORTABLE OUTCOMES

A grant application has been submitted to the DOD CDMRP program in ovarian cancer for continued funding on this highly innovative approach to imaging and therapy of ovarian cancer. In addition grant applications for NIH funding to explore the use of riboswitch technology as we have proposed to image and treat cancer cells using radiolabeled antipyrine have been submitted. Two manuscripts are now in preparation to describe our work on the selection of antipyrine aptamers and the effects of the EpCAM aptamer/antiVICKZ DNA on cell motility.

CONCLUSION

We have selected both DNA and RNA aptamers to antipyrine and derivatives of antipyrine. We have also succeeded in showing cell internalization and functional ability of a large portion of the proposed riboswitch. After design of the folding of the riboswitch, we will be in a position to test the ability of the riboswitch to selectively bind radiolabeled targets composed of antipyrine or antipyrine derivatives. Finally this will permit us to test the ability of the cell internalized riboswitch to bind the radiolabeled targets for imaging and cell killing of ES2 ovarian cancer cells with low background uptake and low toxicity to normal cells..

As an unanticipated byproduct of our work we have found that a DNA construct consisting of an EpCAM aptamer joined to an oligonucleotide complementary to VICKZ mRNA was able to dramatically inhibit motility of ovarian cancer cells compared to untreated cells. Apparently the EpCAM aptamer portion of the construct attaches to its target on the cell surface. When the EpCAM is normally recycled into the cell interior it also internalizes the attached construct. The antiVICKZ portion of the construct then hybridizes to the VICKZ mRNA, making a DNA:RNA hybrid. The RNA portion of the hybrid is then destroyed by the normal intracellular RNase H, resulting in decreased cell motility because of the resulting diminished VICKZ protein required for cell motility. We intend to exploit this novel finding to determine whether this effect can be exploited to restrain ovarian cancer cell metastases in animal models.

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APPENDIX

Statement of Work

Task 1. Optimizing the T3 aptamer.

A number of aptamers that bind T4 have been isolated by the SELEX process. In order to identify the aptamer most likely to be effective in the context of the CO constructs, we will test the ability of these aptamers to bind T3, first in vitro and then in OC cells. These experiments will be performed by the group in Jerusalem.

- 1a. Double aptamer (DA) constructs will be built, consisting of an EpCam aptamer fused to a T3/T4 aptamer. ^{32}P -labeled DA constructs, will be synthesized with a biotin moiety at the 5' end, and the DAs will then be tested for their ability to bind T3-sepharose columns in vitro. These experiments should identify the shortest, effective T3 aptamer that can effectively bind T3 in vitro. (timeframe: months 1-3)

Task 2. Calibrating I^{125} -T3 uptake into, and release from, OC cells.

In parallel to identifying the optimal T3 aptamer, the Boston group will determine the kinetics of I^{125} -T3 uptake into ES2 cells. We will examine the kinetics of T3 cell uptake and discharge, and the binding to normal cell nuclear receptors, since these kinetics will determine the background to be expected after addition of CO and radiolabeled T3.

- 2a. ^{125}I T3 will be added to ES2 cells. At various times, cell activity will be determined. (timeframe, months 1-2)
- 2b. Once we have determined the time to reach steady state levels, the time it takes for the I^{125} -T3 to be released from the cells will be followed. Cells will be incubated with ^{125}I -T3 for the time determined to reach a steady state level and then they will be counted at various times to determine the washout rate of the T3. (timeframe, months 3-4)
- 2c. One of the sources of the background level of T3, is the binding of T3 to normal cell nuclear receptors. In our experiments it may be necessary to block these receptors by addition of blockers such as reverse T3 or tetraiodothyroacetic acid. These blockers will be added at various concentrations to ES2 cells for various times, followed by incubation with ^{125}I T3 to determine the effect of blocking the normal T3 receptors. (timeframe, months 5-6)

Task 3. Optimizing retention within cells of I^{125} -T3 by the EpCam/T3 DA. (timeframe, months 7-13)

- 3a. The Boston group will determine the kinetics of ES2 cellular uptake of the EpCAM/T3 DA in comparison with the non EpCAM containing A2780 cells. ^{32}P labeled DA will

be introduced into the cell media and cells solubilized at various times to determine the time to steady state level of the DA. (timeframe, months 7-8)

- 3b. To determine the effectiveness of the EpCAM/T3 DA constructs in cells, the constructs, with an attached biotin group, will be added to the medium of ES2 cells and allowed to be taken up by the cells for the time it was shown to result in a steady state. ^{125}I -T3 will be added to the cells, and after the time required to reach 5% of steady state levels, the cells will be washed, lysed, and incubated with streptavidin beads that will pull down the biotinylated DAs, and any associated T3. The ability to pull down ^{125}I -T3 will be compared to DAs incubated with EpCam-negative cells and DAs fused to a shuffled T3 aptamer sequence, which is incapable of binding T3. (timeframe, months 9-13)

Task 4. Testing an EpCam-Kras-T3 CO.

- 4a The Israeli group will synthesize the EpCAM-Kras-T3 CO using the published sequences that have been shown to work in cells. The molecule will be tested in vitro, comparing synthetically synthesized wild type and mutant Kras mRNAs for their ability to activate T3 binding. (timeframe, months 4-12)
- 4b. The EpCAM-Kras-T3 CO will then be tested by the U.S. group in OC cells that express EpCam and mutant Kras (MDAH2774). Cells will be incubated with the CO and then incubated with ^{125}I -T3, or the two agents incubated simultaneously and the kinetics of uptake of the T3 determined. (timeframe, months 14-19)

Task 5. Creating an EpCam-VICKZ-T3 CO.

The Israeli group will generate this CO in collaboration with our consultant in New York.

- 5a. We will first test the ability of a series of riboswitches recognizing VICKZ3 mRNA to function as beacons in OC cells. The beacons will be designed and tested by the New York consultant. The sequence most effective as a beacon in vivo will be used to synthesize the CO, along the lines of the EpCam-Kras-T3 CO described above. The Israeli group will test and calibrate the CO in vitro. (timeframe, months 13-24)
- 5b. The U.S. group will then test the EpCAM-VICKZ-T3 CO in ES2 cells, using ^{125}I -T3. Cells will be incubated with this CO and either simultaneously or later incubated with the radiolabeled T3 and then the radioactivity bound to the CO determined. The whole cells will be dissolved and counted. Separately cells incubated with biotin labeled CO will be lysed, incubated with streptavidin beads and counted to determine activity bound to the CO only. (timeframe, months 20-24).

